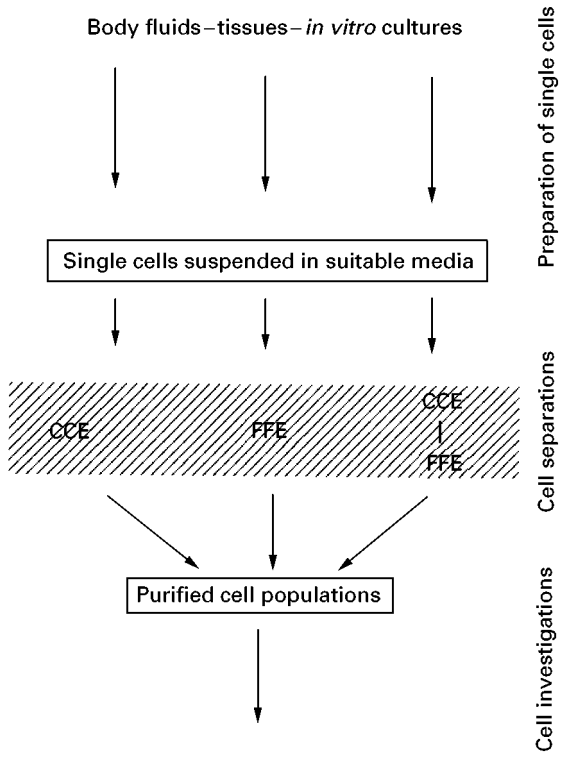

Figure 4 Flow diagram showing a survey of the processes of cell purification described in this article.

differentiation, even if appropriate antibodies are not available.

Cells purified without biological modifications may be especially useful if it is of interest to study their original *in vivo* status or to use them for transplantation purposes and if size or surface charge-related phenomena are to be investigated. As knowledge of possible cellular characteristics and components is continuously accumulating, questions on their actual expression under normal and pathological conditions will frequently arise. For studying such questions, homogeneous cell populations retaining their original *in vivo* status may become so important that techniques and instruments required for their purification will be further improved.

See also: **Cells and Cell Organelles: Field Flow Fractionation.**

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ESSENTIAL GUIDES FOR ISOLATION/ PURIFICATION OF DRUG METABOLITES

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Metabolite Isolation and Identification

Following the administration of drugs to either animals or man, very few of the drugs are excreted unchanged. The majority undergo biotransformations by interaction with a complex series of enzymes. This process, known as drug metabolism, is not restricted to drugs but occurs with all chemicals that are taken in by living systems, including food additives, pesticides, carcinogens, etc. These chemicals are termed exogenous compounds, as opposed to endogenous, or naturally present, compounds.

Metabolic studies have made, and continue to make, fundamental contributions to the drug

discovery process and also to the elucidation of mechanisms of both drug action and toxicity. During the early stages of drug development, an evaluation of the metabolic dispositional profile of a compound may yield valuable information and significantly contribute to the drug candidate selection procedure. In addition, drug metabolism has a central role in the safety evaluation of novel drug substances, and the regulatory authority guidelines for toxicity testing all make reference to metabolic and pharmacokinetic data.

The reactions of drug metabolism may be divided into two groups, the phase I or functionalization reactions and the phase II or conjugation reactions. The phase I reactions involve either the introduction or unmasking of a functional group, e.g. hydroxyl, carboxyl or amino group, within a molecule by the processes of oxidation, reduction or hydrolysis. The groups introduced generally result in an increase in the polarity, and therefore the aqueous solubility, of the metabolite compared with the parent compound. Depending on the reaction type, the change in physicochemical properties may be relatively minor, e.g. dealkylation of a tertiary to a secondary amine, or substantial, e.g. hydrolysis of an ester or amide.

The phase II reactions are biosynthetic and involve the addition of an endogenous molecule to the drug, or a phase I metabolite of the drug, by reaction with a suitable functional group, e.g. carboxyl, hydroxyl, amino, etc. The products of these reactions are generally polar, hydrophilic molecules that are ionized under physiological conditions, and hence the excretion of the foreign compound into urine or bile is facilitated. Examples of reaction types include conjugation with glucuronic acid, sulfate, glutathione and amino acids, all of which result in an increase in the polarity of the product compared to the drug. Some conjugation reactions, namely methylation and acetylation, may result in an increase in the lipid solubility of the metabolite compared that of the drug; however, this depends very much on the nature of the substrate. The two phases of drug metabolism are intimately linked, as shown in **Figure 1**.

As a result of the metabolic transformations outlined here, and analytical sample of biological origin may contain several substances which vary markedly in their physicochemical properties. For example metabolic products may be acidic, basic, neutral or zwitterionic and relatively hydrophilic or hydrophobic. The examination of such samples therefore presents the bioanalyst with a considerable challenge as the sample will contain relatively small quantities of structurally related materials dispersed in an extremely complex matrix containing numerous potentially interfering endogenous materials. The isolation and characterization of metabolic products therefore requires a range of analytical methodologies, primarily in the areas of separation and spectroscopic techniques.

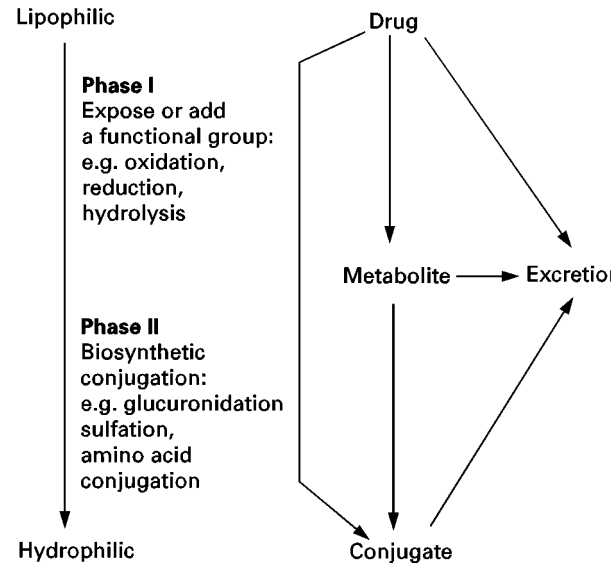

Figure 1 Relationships between phase I and phase II metabolic transformations.

Sample Types

The range of techniques used to investigate the metabolism of drugs is relatively wide (**Table 1**) and the bioanalyst may therefore be presented with samples which vary markedly in terms of both their nature and origin. In *in vivo* metabolic studies, following the administration of a drug to either animals or humans, the major sample types examined include blood, plasma, urine, faeces and less commonly bile and milk. In *in vitro* methodology, sample types may

Table 1 Biological techniques used in drug metabolism

Methodology	*Example*
Administration of the drug to whole animals	Human or standard laboratory species, e.g. rat, dog, etc.
Isolated perfused organs	Liver, kidney, lung, intestine
Tissue slices	Liver, kidney
Isolated cells	Hepatocytes, renal cells, lung cells, enterocytes, blood cells
Subcellular fractions	Whole tissue homogenates, postmitochondrial supernatant, microsomal fractions, cytosol
Purified enzymes	Cytochrome P-450 and flavin monooxygenases

range from relatively clean perfusion fluids to complex tissue homogenates. Thus the bioanalyst may be presented with liquid, semisolid and solid samples for evaluation, each of which presents different problems. Plasma, for example, contains relatively high concentrations of proteins which may interfere with the chromatographic separation of metabolites or damage chromatographic stationary phases. The samples therefore require deproteination prior to analysis. Samples which are solids, or semisolids, may affect the separation characteristics of solid-phase extraction cartridges and it is frequently the case that such samples are homogenized prior to analysis.

Preliminary Sample Pretreatment

Because of the nature of the sample types and the potential range of physiochemical properties of the analytes, the samples encountered in metabolic studies generally require extensive pretreatment prior to instrumental analysis. It is essential that any manipulations carried out on the sample do not result in *ex vivo* changes to the analytes. A general approach to sample treatment is presented in **Figure 2**. Preliminary sample preparation plays an important role in the specificity of an analytical procedure. The initial step involves sample clean-up to remove potentially interfering substances, fractionation of the metabolic products according to their physicochemical properties, concentration of the sample for analysis and possible hydrolysis of conjugated metabolites. Having obtained a primary extract, the analytes are further purified, generally by a chromatographic procedure, prior to characterization by conventional spectroscopic techniques.

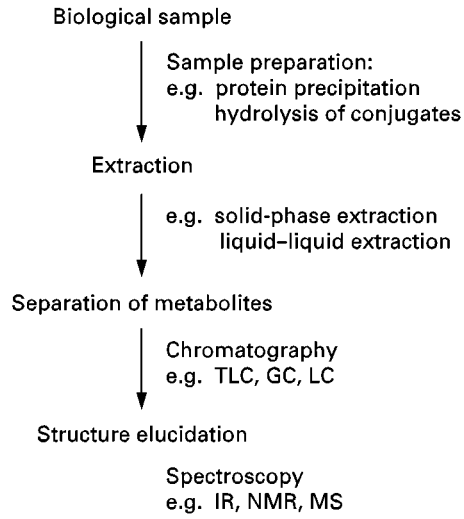

Figure 2 General approach for the isolation and characterization of metabolic products.

Hydrolysis of Conjugates

One of the most frequently encountered phase II pathways is conjugation with glucuronic acid. Several functionalities undergo this reaction, yielding a variety of bond types between the sugar moiety and the aglycone, e.g. carboxyl groups yield acyl glucuronides, phenolic and alcoholic hydroxyl groups yield ether linkages, thiols and amino functions yield *S*- and *N*-glucuronides respectively, and examples of carbon glucuronides are also known. The stability of these various linkages varies considerably, as does their susceptibility to hydrolytic treatments, the common methods of hydrolysis being mild alkali, acid or treatment with the enzyme β-glucuronidase.

A number of problems may occur during sample pretreatment; acyl glucuronides for example are relatively labile under mild alkaline conditions and may undergo hydrolysis in samples which are not stored with care. An additional problem with compounds of this type is that they also undergo facile intramolecular rearrangement at mild alkaline pH giving rise to mixtures of the corresponding 2-, 3- and 4-*O*-acyl esters of glucuronic acid. Such glucuronic acid esters are resistant to hydrolysis by β-glucuronidase but may be hydrolysed by treatment with mild alkali. Thus the amount of aglycone liberated by treatment of the conjugate with the enzyme may be lower than that found following treatment with alkali. Ethereal glucuronides are stable to treatment with mild alkali, but may be hydrolysed with acid or β-glucuronidase. The stability of both *N*- and *S*-glucuronides to either chemical or enzymatic treatment is highly dependent on the nature of the aglycone and the bond type. Carbon-linked glucuronide conjugates are resistant to β-glucuronidase.

The liberation of an aglycone upon incubation of a conjugate with β-glucuronidase may only be taken as presumptive evidence that the conjugate is a glucuronide if adequate controls have been carried out, e.g. inhibition of hydrolysis by the specific β-glucuronidase inhibitor, saccharo-1,4-lactone, identification of the carbohydrate moiety by chromatography and detection with naphthoresorcinol.

Sulfation is a relatively common conjugation reaction for phenolic hydroxyls, alcohols and some amino compounds. Sulfate conjugates may be hydrolysed by aryl sulfatases. However, the commercially available preparations may be contaminated with β-glucuronidase, which should be inhibited by the addition of saccharo 1,4-lactone. Acid hydrolysis of solvolysis may also be employed but the reactivity of the conjugates may vary considerably.

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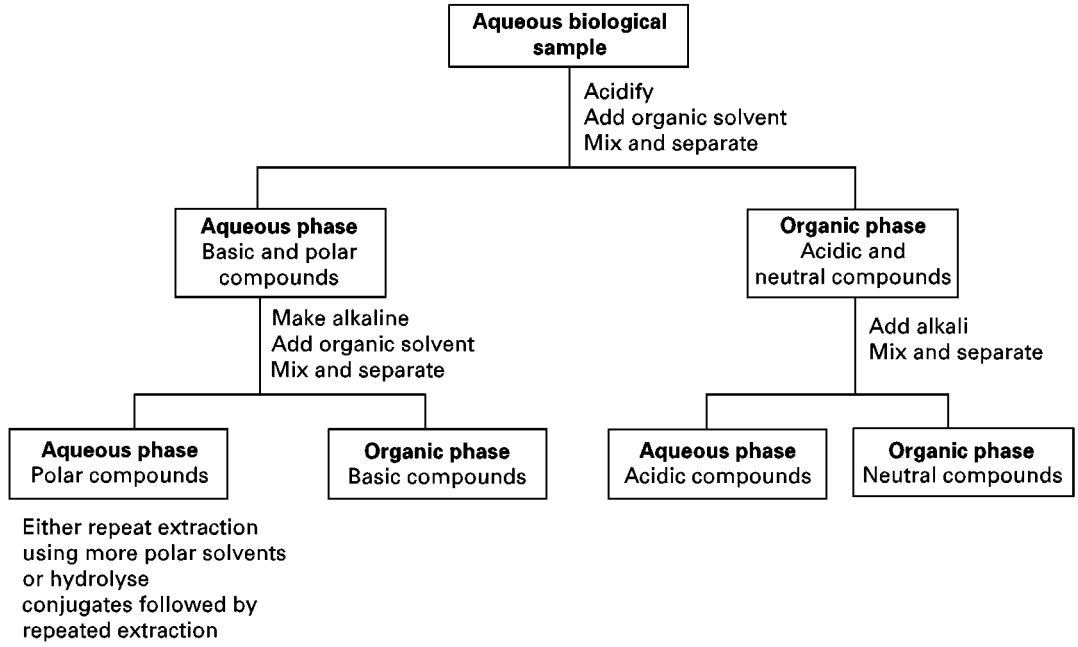

Figure 3 General scheme for the fractionation of drug metabolites using solvent extraction.

In contrast, amino acid conjugates of carboxylic acids are relatively stable and may be isolated and characterized by conventional methodology.

Methods of Isolation – Extraction Techniques

Extraction techniques may be used for the preliminary purification and fractionation of metabolic products from the biological matrix. Further purification of the individual metabolites may be achieved using chromatographic techniques.

Liquid–Liquid Extraction

As pointed out previously, drug metabolites vary greatly in terms of their physicochemical properties. Selective isolation of material may be achieved by extraction from the aqueous-based biological samples, after appropriate adjustment of pH, using an immiscible solvent. The choice of solvent is critical and it may be possible to fractionate the analytes by sequential extraction using solvents of different polarities, with or without adjustment of sample pH. Having obtained an organic extract of the compounds of interest the sample may be cleaned up further by 'back-extraction' into an aqueous phase with appropriate pH adjustment. A general scheme for the extraction of a drug and metabolites is presented in **Figure 3** and examples of commonly used solvents. Used either alone or in combination, are presented in **Table 2**. Thus a particular drug or its metabolites can be selectively isolated from a biological matrix by a consideration of the physicochemical properties of the material, careful choice of solvent and adjustment of the pH of the aqueous medium. Ideally the solvent should completely extract the drug and its metabolites in a single extraction while keeping the amount of coextracted endogenous compounds to a minimum. However, the efficiency of the extraction procedure must be investigated and a double or triple extraction coupled with a 'salting out' procedure may be necessary.

Solvents for extraction should be of high purity grade and it is frequently important that they are distilled prior to use. This distillation will ensure that the solvents are free of trace quantities of solutes which on sample concentration may be present in

Table 2 Examples of commonly used solvents for extraction of biological fluids

Solvent	*Dielectric constant*	*Boiling point (°C)*
n-Hexane	1.9	68.7
n-Heptane	2.0	98.4
Carbon tetrachloride	2.2	76.8
Toluene	2.4	111.0
Diethyl ether	4.3	34.5
Chloroform	4.8	61.2
Ethyl acetate	6.0	77.1
Dichloromethane	9.1	40.2

concentrations greater than that of the analytes and may therefore interfere with the subsequent analysis. Phthalates for example are universal contaminants and are frequently observed in the mass spectra of metabolic products. Solvents such as diethyl ether frequently contain aldehydic impurities which may react with the analyte, e.g. (−)-ephedrine reacts with the acetaldehyde, propionaldehyde or formaldehyde present in ether to yield a series of oxazolidine derivatives during isolation. Chlorinated solvents should be used with caution in the presence of basic compounds; several analgesic agents, e.g. dextromethorphan, pethidine and methadone, have been shown to undergo alkylation during the concentration of dichloromethane extracts of the drugs. Traces of peroxides rapidly form in diethyl ether and may oxidize drugs, or their metabolites. The products of such reactions may then be erroneously identified as metabolites. Additional problems may also arise during concentration of extracts because of the degradation of thermolabile compounds and the loss of volatile compounds. Evaporation under reduced pressure and/or freeze-drying are useful alternative approaches.

Ion Pair Extraction

Highly polar metabolites cannot usually be extracted efficiently using solvents. In this case the metabolites, in their ionized state, can be paired with a counterion of opposite charge. Isolation of several biogenic amines, together with some of their metabolites, has been achieved by this approach. Catecholamines and their derivatives, for example, have been extracted from biological samples using di(2-ethylhexyl)phosphoric acid as a counterion.

Liquid–Solid Extraction

The isolation of drugs and their metabolites by adsorption methods offers an alternative approach to the traditional solvent extraction. Liquid–solid extraction, also known as solid-phase extraction (SPE) employs a wide range of materials including coated charcoal, silica, alumina and ion exchange resins. The biological fluid is passed through a column packed with the adsorbent and the materials of interest are separated from the components of the matrix by elution with an appropriate solvent. The success of the technique depends on the affinity of the analytes for the adsorbent and the strength of the eluting solvent.

In recent years the development of chemically bonded silica stationary phases for liquid chromatography (LC) has resulted in cartridge forms of these materials for liquid–solid extraction and SPE. The use of these packings for extraction is based on the principles of LC; the packing materials however are of larger particle size (50 μm) than those used in LC. Thus analytes with a greater affinity for the stationary phase are retained and highly polar endogenous components of the matrix may be eluted with polar solvents. The retained materials may then be selectively eluted from the packing depending on their physicochemical properties, the nature of the adsorbent and the elution solvent. The range of phases for SPE is fairly extensive and includes C_2, C_8 and C_{18} alkyl, phenyl, cyclohexyl, amino, diol and cyano, in addition to a variety of ion exchange phases. This range allows considerable versatility in terms of both selectivity and specificity for analyte isolation. SPE is superior in many respects to solvent extraction; it is highly reproducible, efficient and easier to automate, it generates less waste solvent and the only major drawback is the relatively high cost of the cartridges.

Methods of Isolation – Chromatographic Techniques

Chromatographic techniques are extensively used in bioanalysis for the separation, isolation and purification of drugs and their metabolites from biological fluids. Such techniques can provide useful preliminary information concerning the physicochemical properties of the metabolites in relation to those of the drug. Although they give little information concerning specific chemical structures, comparison of the chromatographic properties of an analyte with those of an authentic reference compound may provide sufficient information to establish the identity of a particular metabolic product.

Thin-Layer Chromatography

Thin-layer chromatography (TLC) is relatively cheap, easy to use, rapid and robust. These features account for the widespread use of the technique in metabolic studies, particularly for the isolation and purification of analytes prior to their characterization by spectroscopic techniques. A wide variety of stationary phases is available; these include silica gel, alumina and a number of bonded hydrocarbon phases (e.g. C_2, C_8, C_{18}) for reversed-phase and ion exchange separations. Such phases are coated onto plastic, aluminium foil or glass supports. A recent innovation in TLC stationary phase technology involves the introduction of high performance thin-layer chromatographic (HPTLC) plates which are coated with a layer (200 μm) of 5-μm particle size silica which offers improved performance in terms of resolution and

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speed of chromatographic development. A number of chiral TLC phases have also been introduced. However the application of these phases in bioanalytical studies has been limited.

The chromatographic phases can be prepared with fluorescent indicators which facilitate the detection of ultraviolet absorbing analytes. The visualization of analytes may also be achieved by the use of a wide range of chromogenic spray reagents. The colour reactions observed with appropriate reagents may be of assistance in the determination of class of metabolite, e.g. glycine conjugates yield characteristic red-orange colours on treatment with *p*-dimethylaminobenzaldehyde, naphthoresorcinol is used for the detection of glucuronides, potassium dichromate–silver nitrate for sulphur(II) and ninhydrin for glutathione conjugates.

The main application of TLC in metabolic studies is in the isolation of metabolites, for subsequent identification by spectroscopic techniques. However, it can also be used quantitatively if radiolabelled drugs are used, or by the application of scanning densitometry.

Gas Chromatography

Gas chromatography (GC) is the technique of choice for the separation and determination of volatile, thermally stable and relatively low relative-molecular–mass drugs and metabolites. GC separation may be carried out using either packed or capillary columns. GC columns are usually made of glass, stainless-steel, copper, aluminium or PTFE (polytetrafluoroethylene). However in metabolic studies glass columns are preferred to minimize the potential thermal breakdown of metabolites during analysis. For example, the decomposition of primary and secondary hydroxylamines, formed on metabolic *N*-oxidation of the corresponding amines, takes place readily on the heated surfaces of metal columns.

A wide range of stationary phases is available for GC separation and a variety of solid support materials for packed columns is encountered. The amount of loading of the stationary phase on the support may also vary considerably, thus the number of possible combinations of phase and support are essentially unlimited. Liquid phases based on polymers of poly(ethylene glycol) and dimethylsilicone have been widely used in bioanalysis. The poly(ethylene glycols) are polar and Carbowax 20M is frequently used, both with and without potassium hydroxide, as a stationary phase. Carbowax 20M is a particularly useful phase for analysis of low relative-molecular-mass amines and their metabolites, e.g. amphetamine and related compounds. Problems arise with compounds of greater molecular mass because of the maximum operating temperature of 200°C for Carbowax 20M. In contrast the silicon phases may be used up to *c.* 350°C, and common phases encountered in metabolic studies include OV1 and OV101 polymers and the more polar phenylmethylsilicones OV17 and OV25. The most commonly used support materials in bioanalysis include Chromosorb G and W, Gas Chrom Q, Haloport F and Chromosorb 750. These support materials are not entirely inert and are frequently washed with acid or silanized prior to being coated with the stationary phase. Glass beads may also be used as support material for GC because of their inert nature and they may also be coated with low loadings of stationary phase. *N*-Hydroxyamphetamine, a thermolabile metabolite of amphetamine, was first chromatographed successfully without prior derivatization using a column containing Carbowax 20M (0.2%) coated onto a glass bead support.

Several GC detector systems have been described. However only four types are commonly used in bioanalysis, the flame ionization detector, the electron capture detector, the nitrogen–phosphorus detector, and the mass spectrometer. Of these the mass spectrometer is the most specific and can provide information on the structural features of compounds. The flame ionization detector is the most widely used in metabolic studies, and sample quantities as low as 1 ng can be determined depending on detector design. Electron capture detectors respond to halogenated compounds, and compounds containing nitro groups or conjugated carbonyls, etc., and thus the suitability of this detector for metabolic studies depends on the structure of the analyte. This selectivity, together with sensitivity (detection of 1 pg of material is possible) increases the utility of this system. Derivatization of compounds that do not contain halogens with appropriate reagents frequently yields volatile products which are ideal for analysis by GC using this detector system, e.g. the analysis of debrisoquine and its 4-hydroxy metabolite following derivatization with hexafluoroacetylacetone to yield the corresponding bis(trifluoromethyl)pyrimidine derivatives. Nitrogen–phosphorus detectors are extremely sensitive and are 20 000–40 000 times more sensitive to nitrogen than to carbon. In bioanalysis such detectors are useful particularly for heterocyclic compounds, which are less likely to lose nitrogen via metabolic deamination than are acyclic compounds.

The products of metabolism are generally more polar than the drug, have less volatility, long GC retention times and produce tailing peaks. Thus metabolites are frequently derivatized prior to analysis to increase volatility, modify chromatographic properties and increase detector response. The most common techniques are: silylation, the replacement

of active hydrogen by trimethylsilyl groups (e.g. OH, SH, NH_2); alkylation using diazomethane, dimethylformamide, dialkyl acetals, or boron trifluoride and an alcohol; and acylation using perfluoroacyl reagents to yield trifluoroacetyl, heptafluorobutyryl, etc. derivatives.

Liquid Chromatography

LC is an extremely versatile technique and has a number of advantages over GC in terms of bioanalysis. For example, highly polar compounds which are difficult to extract from aqueous solutions, e.g. glucuronide and sulfate conjugates and quaternary ammonium derivatives, may be analysed directly without the necessity for extraction; the technique is normally carried out at room temperature and therefore thermolability of analytes is not a major consideration; and the technique is nondestructive so that the eluent containing the analytes may be collected and used for additional offline characterization.

A variety of different stationary phases is available for LC, but the reversed-phase packings (e.g. C_2, C_8, C_{18}, phenyl) are the most commonly used in metabolic studies. The mobile phases utilized with such columns are based on aqueous solvents containing variable quantities of organic modifiers. Such systems allow analyte sample preparation to be simplified, reducing the preanalysis manipulation steps. Provided the chromatographic system has sufficient resolving power, it may be possible to inject directly either a dilute sample or a plasma sample following precipitation of plasma proteins with an organic solvent compatible with the LC mobile phase. This approach has the obvious advantages of reduction of tedious sample manipulation steps and also in the reduction of human exposure to samples of clinical origin. A disadvantage of the direct injection approach is that protein precipitation may occur on injection of plasma samples into the chromatograph. However, the use of precolumns can protect the analytical column and the instrument.

The most frequently used detection systems in LC analysis are either fixed- or variable-wavelength ultraviolet (UV) detectors, fluorescence detectors or electrochemical detectors. While analyte derivatization is not as commonly used with LC, as with GC, derivatives may be used to enhance the detector sensitivity, particularly if fluorescence is used. Multiple-wavelength UV detectors and the diode array detector are particularly useful in metabolic studies. The use of such detectors in analysis provides a three-dimensional chromatographic retention and the entire spectrum of a sample may be determined in a single chromatographic run. The main applications of these detector systems in bioanalysis are to ensure chromatographic peak purity and to provide initial spectroscopic data for metabolite identification. If the compound under investigation is radiolabelled then a suitable LC radiodetector may also be used to facilitate the detection of metabolites.

Capillary Electrophoresis

A technique that is likely to make an impact on bioanalysis in the near future is capillary electrophoresis (CE). This technique, and its variants, offers a number of advantages over conventional chromatographic techniques, e.g. high column efficiencies and short analysis times, particularly where samples are complex mixtures. At present CE instruments are limited in terms of sample size, but this may be an advantage when working with biological fluids as it minimizes potential contaminants. An additional advantage of the technique is that by manipulation of the analytical conditions the nature of the components entering the capillary may be controlled and interference effects reduced. A variety of detector systems has been described for CE, including fluorescence, electrochemical and mass spectrometry systems, but the majority of commercially available instruments incorporate a sensitive UV detector system. Bioanalytical applications of CE have been described for the determination of cefpiramide and anticancer agents in human plasma.

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Methods of Identification

The elucidation of metabolite structure is dependent on the use of spectroscopic techniques, either directly linked to chromatographic systems (the so-called 'hyphenated': techniques, GC-MS, LC-MS), or used offline following the isolation and purification of analytes. The main techniques used in bioanalysis are ultraviolet (UV), infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). However, the technique or combination of techniques finally adopted will be dependent on the complexity of the problem and the amount of pure isolated material available.

Ultraviolet Spectroscopy

This technique is widely used for the quantitative analysis of drugs and metabolites in biological samples. However, the amount of structural information which can be obtained from a UV spectrum of a metabolite is limited. If the site of metabolism in a molecule is at, or adjacent to, a chromophore then the UV spectrum is likely to yield useful structural information, e.g. oxidation of an aromatic ring to

yield a phenol, the spectrum of which can be influenced by alteration of pH, reduction of an aromatic nitro group to yield an amine, and reactions which result in the introduction of conjugated double bonds, e.g. aromatization.

Infrared Spectroscopy

IR spectra are highly diagnostic and the examination of an IR spectrum provides a simple, rapid and often reliable method of assigning metabolite structure. Until relatively recently the sensitivity of IR spectrometers restricted their application in metabolic studies but the development of Fourier transform infrared (FTIR) and the application of these instruments as detector systems for both GC and supercritical fluid chromatography has increased the potential of the technique in bioanalysis significantly.

Nuclear Magnetic Resonance Spectroscopy

NMR spectroscopy has been routinely used in metabolic studies as a means of structure elucidation for a number of years. The major limitation of the technique has been sensitivity. However instrumental developments, with improvements in resolution, analytical power and sensitivity, have changed the way the technique is used in bioanalysis.

There are now a number of reports of direct NMR examination of biological samples with minimal or no preliminary sample clean-up processes and biological fluids have been placed directly in the NMR sample tube. It has also been possible, using NMR techniques, to examine metabolism and distribution in cells and organs.

Proton NMR is the most widely used technique in drug metabolism because of its high sensitivity and the large number of observable protons in most drugs and their metabolites. As bioanalytical samples are initially obtained in aqueous solution the intense water signal present must be either eliminated, suppressed or edited out of the spectrum. Signals from endogenous materials may also obscure signals of interest because of overlap resulting from the narrow chemical shift range in proton NMR. Direct proton NMR has been used to examine the urinary disposition of paracetamol following the oral administration of the drug to humans. Using this approach it was possible to examine the urinary metabolite profile following both therapeutic doses and overdoses of the drug.

Other nuclei of interest in bioanalysis include ^{19}F, ^{31}P, ^{15}N and ^{13}C, although sensitivity may be a problem with some of these nuclei (e.g. ^{15}N). An approach which may yield useful information with these nuclei is to use compounds appropriately labelled with stable isotopes. This approach may be particularly useful if the label is introduced at a site which undergoes transformation. For example the fate of [carboxyl-^{13}C]phenylacetic acid has been examined following administration to a horse. This compound undergoes both amino acid and glucuronic acid conjugation and the major metabolites could readily be distinguished following direct NMR examination of urine samples and observation of the ^{13}C-carbonyl resonances. NMR has also been used directly linked with HPLC for metabolite work.

Mass Spectrometry

As a result of its extreme sensitivity and ability to provide diagnostic information, MS is the standard technique for the identification of metabolic products. All the major MS techniques have been utilized in metabolic investigations and thus it is relatively easy to find publications detailing applications of electron impact, chemical ionization, field desorption and fast atom bombardment in drug metabolism. The major advantage of MS is the ability to use the technique in combination with either GC or LC. Such hyphenated techniques, particularly GC-MS, have been extensively used in metabolic studies and the development of thermospray ionization for LC-MS has enabled spectra of nonvolatile hydrophilic analytes, e.g. metabolic conjugates, to be determined directly. As a result of the variety of ionization modes available, and the ability to link the technique to GC or LC, there are few bioanalytical problems to which MS cannot make a valuable contribution.

There are essentially three main applications of MS, particularly the hyphenated techniques, in metabolic studies.

1. The characterization and structure elucidation of metabolic products.
2. Quantitative analysis using the mass spectrometer as a sensitive chromatographic detector for selected single or multiple ion monitoring, using for example compounds labelled with stable isotopes as internal standards. An alternative approach involves the administration of a labelled compound to a patient who is at steady state, and use of the nonlabelled material to examine the pharmacokinetics of what is effectively a 'single' drug dose under these conditions.
3. Mechanistic investigations, e.g. the source of oxygen in a metabolite may be determined by carrying out appropriate experiments using $^{18}O_2$ or $H_2{}^{18}O$; the use of compounds labelled with stable isotopes, e.g. replacement of hydrogen with deuterium to determine kinetic isotope effects on metabolism.

MS has made a number of important contributions to drug metabolism and the further development of the technique, together with advances in instrumentation, will enhance its application in this area.

See also: **II/Chromatography: Gas:** Column Technology; Detectors: Mass Spectrometry. **Chromatography: Liquid:** Detectors: Ultraviolet and visible Detection; Nuclear Magnetic Resonance Detectors. **Chromatography: Thin-Layer (planar):** Layers. **Electrophoresis:** Capillary Electrophoresis; Capillary Electrophoresis Detection; Capillary Electrophoresis-Mass Spectrometry; Capillary Electrophoresis-Nuclear Magnetic Resonance. **Extraction:** Analytical Extractions; Solid-Phase Extraction; Solvent Based Separation; **III/Drugs of Abuse: Solid-Phase Extraction. Drugs and Metabolites:** Liquid Chromatography-Mass Spectrometry; Liquid Chromatography-Nuclear Magnetic Resonance-Mass Spectrometry.

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ESSENTIAL GUIDES FOR ISOLATION/ PURIFICATION OF ENZYMES AND PROTEINS

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Nature of the Problem

The purification of proteins presents a unique challenge in the field of separation science. Typically, the particular protein to be isolated will constitute 1% or less (sometimes much less) of the material in the original extract and all of the contaminants will have basically the same chemical characteristics, i.e. they are all proteins. There is the added complication that for most purposes it is necessary to retain the biological activity of the protein, and the inherent instability of protein structures restricts the range of temperatures and solvent compositions that can be used during purification.

Tools for its Solution

Clearly, methods for the separation of proteins must be based on those characteristics in which they differ from one another. The most important of these are listed in **Table 1** along with the separation techniques that exploit those differences. These various properties are not of equal generality or of equal utility for purification purposes.

By far the most widely used technique for protein isolation is ion exchange chromatography. The generality of the method arises from the fact that proteins contain ionizable amino acids and hence carry a net charge at all pH values except the unique pH (the isoelectric point) at which the positive and negative charges are equal. Moreover, two proteins that have the same charge at a particular pH are likely to differ in charge at some other pH. Ion exchange chromatography is technically simple and can be adopted for use over a very large range of scales. Chromatofocusing and isoelectric focusing are methods that depend on the differences in isoelectric points between proteins but are less widely used for preparative work than is ion exchange chromatography because of increased cost, restrictions of scale and technical difficulty.

Electrophoresis is a special case. Electrophoretic methods are of central importance in analytical protein chemistry but, until recently, have not proved

Table 1 Properties of proteins that can be exploited for purification and associated experimental methods

Electrical charge	Ion exchange chromatography Chromatofocusing Isoelectric focusing (Electrophoresis)
Hydrophobic surface regions	Hydrophobic chromatography
General surface properties	Salt fractionation
Size	Size-exclusion chromatography Membrane filtration
Specific binding site	Affinity chromatography Dye-binding chromatography
Surface carbohydrate	Lectin chromatography
Metal-binding site	Metal chelate chromatography
Antigenic determinants	Immunoaffinity chromatography

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